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(54) Title: TREATMENT OF OSTEOPOROSIS WITH LEPTIN			
(57) Abstract			
<p>Methods for inducing differentiation of a bone marrow stromal progenitor cell to an osteoblastic lineage are described. Methods for increasing bone formation in a mammal also are described.</p>			

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## **TREATMENT OF OSTEOPOROSIS WITH LEPTIN**

### **Background of the Invention**

5           Obesity and osteoporosis are two major causes of health expenditures in Western societies. These conditions, however, appear mutually exclusive since body weight is one of the strongest predictors of the bone mineral density (BMD) in both sexes. Several recent studies have pointed out that body fat and lean body mass (LBM) play specific roles in the relationship between body weight and BMD.

10   Body fat has been reported to be a more important explanatory factor for hip fracture than body weight and LBM. It has been suggested that fat mass plays a role at trabecular bone sites, independent of whether the site it is weight-bearing or not. Therefore, this positive fat mass effect on BMD can be dissociated from a single weight-loading effect. Adipose tissue produces estrogens by androgen

15   aromatization, and represents the principal source of estrogens after menopause. Androgen conversion by adipose tissue has been widely accepted as the major endocrine factor explaining the protective effect of obesity on bone. Nevertheless, fat mass and BMD are positively and strongly correlated, even after normalization by serum estrogen levels. Furthermore, fat mass or body weight and BMD are

20   correlated in women independently of their menopausal status, suggesting that other humoral or metabolic factors should be involved in the mechanism of fat-induced bone protection.

### **Summary of the Invention**

          The invention is based on the discovery that leptin can be used to induce

25   differentiation of a bone marrow stromal progenitor cell to an osteoblastic lineage and to induce bone formation in mammals. Thus, the invention provides new methods for treating osteoporosis, as well as other conditions where bone formation is desired. In addition, the invention is based on the discovery that administration

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of a combination of estrogen and leptin to a mammal decreases bone turnover more effectively than administration of estrogen alone.

The invention features a method of inducing differentiation of a bone marrow stromal progenitor cell to an osteoblastic lineage. The method includes  
5 contacting a bone marrow progenitor cell with an amount of leptin or an analog thereof effective to induce differentiation of the bone marrow progenitor cell. The method also can include the step of monitoring markers of osteoblast differentiation.

As used herein, leptin includes recombinant leptin (rh-leptin), chemically  
10 synthesized leptin, or leptin purified from a natural source. Analogs of leptin include (1) fragments of leptin, (2) leptin having amino acid substitutions, deletions or additions, (3) synthetic peptides, and (4) chemically modified leptin, such as leptin containing additional material functioning as carrier to prevent degradation of the therapeutically effective portion, all of which are substantially homologous with  
15 or correspond to the sequence of native leptin. Leptin analogs appropriate for use in the present invention induce bone formation and have osteoblast differentiation activity.

The invention also features a method of inducing bone formation in a mammal. The method includes administering an amount of leptin or an analog  
20 thereof effective to induce bone formation in the mammal. The method further can include monitoring markers of osteoblast differentiation, or monitoring beneficial increases in bone mineral density or bone structure in the mammal. Leptin or an analog thereof can be administered intravenously, intraperitoneally, intrathecally, subcutaneously, intramuscularly, intranasally, orally, through inhalation or by  
25 suppository, depending on various patient indications. The method further can include administering estrogen with leptin or an analog of leptin.

The invention also features an article of manufacture that includes packaging material and a pharmaceutical agent within the packaging material. The pharmaceutical agent is therapeutically effective for treating osteoporosis in a  
30 patient and the packaging material includes a label that indicates that the

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pharmaceutical agent is useful for treating osteoporosis in a patient. The pharmaceutical agent includes leptin or an analog thereof, and further can include estrogen. Use of leptin or an analog thereof in the manufacture of a medicament for the treatment of osteoporosis, and pharmaceutical compositions that include  
5 estrogen, leptin or an analog thereof, and a pharmaceutically acceptable carrier also are featured.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or  
10 equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only  
15 and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### **Brief Description of the Drawings**

Figure 1 is a graph that depicts the effects of leptin administration on  
20 proliferation of hms[2-12] cells at 34°C and 39.5°C, as assessed by thymidine-incorporation. The solid bar is in the absence of leptin and the open bar is the presence of 0.6 µg/ml leptin.

Figures 2A-2C are graphs that depict alkaline phosphatase, type I collagen, and osteocalcin gene expression, respectively, as measured by RT-PCR, in  
25 hms[2-12] cells after 3 days (Figures 2A-2B) or 6 days (Figure 2C) in the presence of vehicle (solid bar) or in the presence of 0.15, 0.3, 0.6, 1.2, or 2.4 µg/ml of leptin (open bars).

Figures 3A-3C are graphs that depict lipoprotein lipase (LPL), adiponin, and leptin gene expression, respectively, as measured by RT-PCR, in hms[2-12]

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cells after 3 days (Figure 3A) or 9 days (Figure 3B-3C) in the presence of vehicle (solid bar) or in the presence of 0.15, 0.3, 0.6, 1.2, or 2.4  $\mu\text{g/ml}$  of leptin (open bars).

Figures 4A and 4B are graphs that depict AP activity in hms[2-12] cells.

- 5 In Figure 4A, cells were incubated for 3, 6, and 9 days in the presence of vehicle (solid squares) or in the presence of 0.6  $\mu\text{g/ml}$  leptin (open squares). In Figure 4B, cells were incubated for 3 days in the presence of vehicle (solid bar) or 0.075, 0.15, 0.3, 0.67, 1.2, or 2.4  $\mu\text{g/ml}$  of leptin (open bars).

- Figures 5A and 5B are graphs that depict type I collagen and osteocalcin  
10 secretion in hms[2-12] cells cultured for 12-21 days at 39.5°C in the presence of vehicle (solid squares) or in 0.6  $\mu\text{g/ml}$  leptin (open squares).

Figure 6 is a graph that depicts mineralization of hms[2-12] cells in the absence (solid bars) or in the presence of 0.6  $\mu\text{g/ml}$  leptin (open bars) after 12-21 days in culture at 39.5°C.

- 15 Figure 7 is a graph that depicts triglyceride accumulation in hms[2-12] cells in the presence of vehicle (solid bars) or in the presence of 0.6  $\mu\text{g/ml}$  leptin (open bars) over 6-15 days in culture at 39.5°C.

Figure 8 is a graph that depicts daily weight gain of animals after OVX.

- Figure 9 is a graph that indicates average food intake of animals after  
20 OVX.

Figure 10 is a graph that indicates cancellous bone volume of animal treatment groups.

Figure 11 is a graph that indicates trabecular bone volume of animal treatment groups.

- 25 Figure 12 is a graph that indicates bone formation rate of animal treatment groups.

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### **Detailed Description**

#### *Inducing Differentiation of Bone Marrow Stromal Progenitors to Osteoblastic Lineage*

In one aspect, the invention features a method for inducing  
5 differentiation of a bone marrow stromal progenitor cell to an osteoblastic lineage. The method includes contacting a bone marrow stromal progenitor cell with an amount of leptin or an analog thereof, effective to induce differentiation to an osteoblastic lineage. The method also can include the step of monitoring markers of osteoblast differentiation.

10 Leptin is the 16 kD gene product of the *ob* gene which, when mutated, results in obesity. Leptin is secreted mainly by white adipose tissue and regulates food-intake and body weight by negative feed-back on the hypothalamic nuclei. In addition to its action in the central nervous system, leptin also influences glucose metabolism and suppresses specific biochemical reactions that contribute to lipid  
15 accumulation and cell differentiation in peripheral tissues such as lung, kidney, fat, and muscle, where leptin receptor has been described.

Suitable bone marrow stromal progenitor cells include without limitation, conditionally immortalized human marrow stromal (hMS) cell lines. The hMS(2-12) cell line is particularly suitable for this method. This cell line was  
20 established by transfecting hMS cells with a gene encoding temperature-sensitive mutant TsA58 of SV40 large T antigen (SV40LTA). Hicok, K. et al., J. Bone Miner. Res., 1998, 13:1-13. At the "permissive" temperature of 34°C, cell proliferation is increased and differentiation is inhibited until confluence. At the "restrictive" temperature of 39.5°C, the SV40LTA is consistently inactive, little cell  
25 division occurs, allowing differentiation to be studied with an essentially clonal population of normal human marrow stromal cells. Thus, it is possible to regulate the immortalization process by a temperature switch from 34°C, at which cells are maintained in a conditionally immortalized state, to 39.5°C, at which cells express a normal phenotype.

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The bone marrow stromal progenitor cells are thought to be targets of leptin, as the OB-R gene for the long splice variant of the leptin receptor (leptin-R) is expressed, and leptin-R protein is present, in these cells. The long splice variant of the OB-R gene was expressed only when cells were cultured at the restrictive temperature, and this was confirmed at the protein level. The short form of the leptin-R was present constitutively at the permissive temperature, and its expression was not regulated by the temperature switch. Leptin administration did not modulate gene expression and protein level of either form of the OB-R. DNA synthesis, as assessed by [<sup>3</sup>H]-thymidine incorporation, was not increased in leptin-treated cells. The absence, or the very low presence, of the long form of leptin-R, may have contributed to this lack of proliferative effect at 34°C. Overexpression of the LTASV40 may mask moderate proliferative effects of leptin in this cell line at the permissive temperature. It is unlikely that phenotypic differences induced by leptin administration were related to an increased number of osteoblastic progenitors.

Markers of osteoblast differentiation can be monitored by examining expression of genes encoding osteoblastic markers, or by examining levels of protein or protein activity. Osteoblast differentiation markers include alkaline phosphatase, osteocalcin, and type I collagen. In addition, mineralization can be assessed as a marker of osteoblast differentiation.

Bone marrow stromal progenitor cells contacted by leptin exhibit a dose-dependent increase in osteoblast differentiation. This effect appears to be at the level of maturation, rather than at the level of commitment. No early or late effects of gene expression were observed for core-binding factor  $\alpha 1$  (*Osf2/Cbfa1*), a transcription factor involved in commitment to the osteoblast differentiation pathway. Rodan, G.A. et al., *Cell*, 89:667-680 (1997). Mice with targeted disruption of *Osf2/Cbfa1* have a cartilaginous skeleton that cannot mineralize. In contrast, consistent dose-dependent changes in steady state levels of mRNA and protein markers of osteoblast differentiation, including alkaline phosphatase, osteocalcin, and type I collagen, were observed with leptin treatment. Moreover,



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- leptin treatment directly increased mineralization, the hallmark of the osteoblast phenotype. Concordant with the data described herein, a recent paper showed a correlation of leptin level and bone area in pubertal girls. Matkovic, V., et al., J. Clin. Endocrinol. Metab., 1997, 82:3239-3245. This could be interpreted as a
- 5 positive effect of leptin on mesenchymal cells resulting in an increased periosteal apposition. A recent preliminary report indicated that leptin administration stimulated bone formation in leptin deficient mice (ob/ob). Liv, C., et al., J. Bone Miner. Res., 1997, 12 (Suppl. 1), 5115. Numerous metabolic impairments were corrected, however, by administration of leptin to these animals.
- 10 Without being limited to particular mechanisms, leptin could increase osteoblastic differentiation through the Jak/STAT kinase cascade. The leptin-R is closely related to gp130 protein, and ligand binding of gp130 stimulates phosphorylation of the Jak/STAT kinase cascade. Oncostatin M and leukemia inhibitory factor are gp130-dependent inducers of osteoblastic gene transcription.
- 15 Jay, P.R. et al., Endocrinology, 137:1151-1158 (1996); and Levy, J.B. et al., Endocrinology, 137:1159-1165 (1996).
- Administration of leptin to bone marrow stromal progenitor cells resulted in a decrease in neutral lipid accumulation, as well as in adipsin and leptin mRNA steady-state levels. Expression of the gene encoding peroxisome-
- 20 proliferator-activated receptor  $\gamma$ 2 (PPAR $\gamma$ 2) and levels of PPAR $\gamma$ 2 protein were not changed by administration of leptin. Lipoprotein lipase (LPL), a marker of the early stage of adipocyte differentiation, was increased. The accumulation of cytoplasmic lipid droplets, an adipocyte phenotype, was decreased indicating that leptin decreases adipocyte differentiation. The decrease in neutral lipid
- 25 accumulation is consistent with studies showing that leptin lowers lipogenesis in a preadipocytic cell line and triglyceride accumulation in transfected rat pancreatic islets. Bai, Y. et al., J. Biol. Chem., 271:13939-13942 (1996); and Shimabukuro, M. et al., Proc. Natl. Acad. Sci. USA, 94:4637-4641 (1997). Leptin also can induce an increase in free fatty acid oxidation.

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The reasons for the paradoxical increase in expression of the genes encoding LPL and the decrease in expression of the genes encoding adipsin and leptin are not clear. *In vivo* administration of leptin to rodents also increased LPL gene expression and decreased leptin gene expression. Sarmiento, U. et al., Lab. Invest., 77:243-256 (1997); and Siegrist-Kaiser, C.A. et al., J. Clin. Invest., 100:2858-2864 (1997). It has been shown that overexpression of LPL in transgenic mice did not lead to an increase of fat deposition, but instead resulted in a decrease in plasma triglycerides. Induction of LPL may provide energy for marrow stromal cell differentiation by enhancing hydrolysis of extracellular triglycerides for differentiation pathways. As no changes in PPAR $\gamma$ 2 gene expression and protein level were observed with leptin administration, the inhibition of adipocyte differentiation following leptin administration could be mediated by the phosphorylation of PPAR $\gamma$ 2 and the down-regulation of the gene encoding adipsin, a PPAR $\gamma$ 2-regulated gene. Expression of the gene encoding adipsin is increased during fasting and decreased during hyperinsulinemic states. Furthermore, the demonstration that the gene encoding LPL is unregulated through binding of the PPAR-RXR heterodimer to a functional response element in the human LPL gene promoter suggests a complex interaction between the OB-R and PPAR $\gamma$ 2 signaling pathways in late differentiation.

Thus, because leptin enhances osteoblast differentiation but inhibits adipocyte differentiation, leptin may be a physiologic regulator of the shunting between these two differentiation pathways. As described herein, increased production of leptin may contribute to the increased bone mass observed with obesity. Hence, leptin could adjust bone strength to a stressing situation associated with the increased skeleton loading consequence of obesity and thereby participate in the positive effects of fat on bone.

#### *Production of Leptin or Analogs Thereof*

Leptin can be obtained in various ways, including by recombinant expression, purification from a biological sample, or chemical synthesis. Leptin is

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available commercially, for example, from Amgen, Inc. (Thousand Oaks, CA), R&D Systems (Minneapolis, MN), or Peptotech (Rocky Hill, NJ). Recombinant leptin can be produced by ligating a nucleic acid sequence encoding leptin or an analog thereof into a nucleic acid construct such as an expression vector, and  
5 transforming a bacterial or eukaryotic host cell with the expression vector. The cDNA sequence encoding human leptin has GenBank Accession number U18915 g623331. Zhang, Y. et al., Nature, 372:425-432 (1994).

In general, nucleic acid constructs include a regulatory sequence operably linked to a leptin nucleic acid sequence. Regulatory sequences do not  
10 typically encode a gene product, but instead affect the expression of the nucleic acid sequence. In bacterial systems, a strain of *Escherichia coli* such as BL-21 can be used. Leptin expressed in *E. coli* typically is found in inclusion bodies. Suitable *E. coli* vectors include without limitation the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST) and a thrombin or  
15 factor Xa protease cleavage site, or the pET series of vectors (Novagen, Madison, WI) that produce fusion proteins with a hexahistidine tag and a thrombin cleavage site. Transformed *E. coli* are typically grown exponentially, then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, soluble fusion proteins are purified easily from lysed cells by adsorption to glutathione-  
20 agarose beads followed by elution in the presence of free glutathione or by nickel affinity chromatography. Leptin can be purified from inclusion bodies by washing the inclusion bodies in a buffer containing a non-ionic detergent such as Triton X-100, and solubilizing the remaining inclusion body pellet in guanidine HCl. Affinity chromatography can be used to purify leptin from the solubilized inclusion  
25 bodies, as described above. Leptin can be refolded by step dialysis or direct dilution. See, for example, Fawzi, A.B. et al., Horm. Metab. Res., 1996, 28(12):694-697; and Varnerin, J.P. et al., Protein Expr. Purif., 1998, 14(3):335-342 for a description of production of leptin in *E. coli*.

In eukaryotic host cells, a number of viral-based expression systems can  
30 be utilized to express leptin. A nucleic acid encoding leptin can be cloned into, for

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example, a baculoviral vector and then used to transfect insect cells, such as *Spodoptera frugiperda* (Sf-9) cells. See, for example, Churgay, L.M. et al., Gene, 1997, 190(1):131-137. Alternatively, the nucleic acid encoding leptin can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect

5 host cells.

Mammalian cell lines that stably express leptin can be produced by using expression vectors with the appropriate control elements and a selectable marker. For example, the eukaryotic expression vector pCDNA.3.1<sup>+</sup> (Invitrogen, San Diego, CA) is suitable for expression of leptin in, for example, COS cells or

10 HEK293 cells. Following introduction of the expression vector by electroporation, DEAE dextran, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce leptin. Leptin also can be transcribed and translated *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

15 Leptin also can be purified from a biological sample using standard protein purification techniques such as affinity chromatography, gel-filtration, and ion-exchange chromatography. Leptin analogs can be screened for activity using the methods described herein.

Analogues of leptin with amino acid substitutions, deletions, or additions

20 can be made with standard recombinant techniques. For examples, analogs of leptin with amino acid substitutions, such as conservative amino acid substitution can be used. In general, conservative amino acid substitutions, i.e., substitutions of similar amino acids, are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties. For example,

25 aspartate and glutamate and isoleucine and valine are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. Dayhoff et al., Atlas of Protein Sequence and Structure, 1978, 5(3):345-352, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity.

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Analogs of leptin can be made using standard recombinant techniques, including random and site-directed mutagenesis techniques. See, Chapter 8 of Short Protocols in Molecular Biology, second edition, Ausubel et al., 1992, for a discussion of mutagenesis methods. Specific point changes can be introduced into  
5 the gene encoding leptin by oligonucleotide-directed mutagenesis. In this method, the desired change is incorporated into an oligonucleotide, which then is hybridized to the wild-type nucleic acid. The oligonucleotide is extended with a DNA polymerase, creating a heteroduplex that contains a mismatch at the introduced point change. The mismatch is repaired upon transformation of *E. coli*, and the  
10 gene encoding the leptin analog can be re-isolated from *E. coli*. Kits for introducing site-directed mutations can be purchased commercially. For example, Muta-Gene® in-vitro mutagenesis kits can be purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Random changes can be made by incubating a single-stranded DNA  
15 encoding leptin with chemicals that damage the base moiety of the nucleotide. For example, single-stranded DNA can be incubated with chemicals such as nitrous acid, formic acid, or hydrazine. After performing a primer extension, the DNA encoding leptin is isolated, used to transform *E. coli*, and mutagenized DNA then is recovered from *E. coli*.

20 Clusters of mutations can be introduced, for example, by using the linker scanning method. In this method, a plasmid encoding leptin is linearized near a region of interest, and digested with a nuclease such as Bal 31, or exonuclease III and S1 nuclease. Oligonucleotide linkers containing a restriction endonuclease site and mutated sequence, then are ligated to the digested, plasmid  
25 ends. A complete plasmid is reconstructed by using the backbone from the wild type plasmid and the region of interest from the digested plasmid.

Polymerase chain reaction (PCR) techniques also can be used to introduce mutations. PCR is a procedure in which target nucleic acids are amplified. For random mutagenesis, degenerate oligonucleotides can be used to

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amplify the target nucleic acids. Point mutations also can be introduced by using oligonucleotides that incorporate the desired point change.

*Methods of Inducing Bone Formation in Mammals*

Methods of inducing bone formation in a mammal include administering  
5 an amount of leptin or an analog thereof effective to induce bone formation in the mammal. The method also can include monitoring markers of osteoblast differentiation in the mammal, as described above, or monitoring beneficial changes in bone mineral density or bone structure in the mammal. Bone density and structure can be assessed, for example, by dual energy X-ray absorptiometry,  
10 computerized tomography, single photon absorptiometry, dual photon absorptiometry, or ultrasound techniques. Leptin or analogs thereof are particularly useful for inducing bone formation in human patients with osteoporosis. Osteoporosis is a skeletal condition characterized by a decreased density of normally mineralized bone, leading to an increased risk of fractures. Post-  
15 menopausal, age-related, and idiopathic osteoporosis are non-limiting examples of primary osteoporosis that can be treated with leptin or an analog thereof. Secondary forms of osteoporosis caused by, for example, excessive alcohol intake, hypogonadism, hypercortisolism, and hyperthyroidism also can be treated using this method.

20 The amount of leptin or an analog thereof effective to induce bone formation in a mammal may vary, depending on a number of factors, including the preferred dosage of the compound to be administered, the chemical characteristics of the compounds employed, the formulation of the compound excipients and the route of administration. The optimal dosage of leptin or an analog thereof to be  
25 administered may also depend on such variables as the overall health status of the particular patient and the relative biological efficacy of leptin or analog thereof selected. For example, approximately 300 to 770 µg of leptin per kg body weight can be used.

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Bone mineral density (BMD) increases with fat body mass (FBM), and obesity has a protective effect against osteoporosis. The relationship between FBM and BMD is only partially explained, however, by a combination of hormonal and mechanical factors. Serum leptin levels are strongly and directly related to FBM.

5 As demonstrated herein, leptin is a hormonal mediator relating fat mass and bone mass. In studies reported below, the *in vivo* effects of leptin administration were compared with those of estrogen therapy for 1 month in the prevention of ovariectomy (OVX)-induced bone loss in 6-months old Sprague-Dawley rats. As assessed by histomorphometry, leptin reduced trabecular bone loss and trabecular

10 architectural changes although this effect was not a complete prevention as with estrogen treatment. Leptin treatment depressed periosteal bone formation whereas no difference in indices of bone turnover was observed on cancellous bone surfaces. Leptin may play an important role in inhibiting bone resorption in obese patients and consequently, be a major contributor to the protective effect on obesity

15 in the development of osteoporosis.

A combination of estrogen and leptin further decreased the bone turnover compared to estrogen treated OVX rats and shams operated rats. Thus, methods of the invention include administering estrogen and leptin or an analog of leptin to induce bone formation by modulating bone remodeling in favor of a better

20 bone balance. Estrogen can be administered in the form of, for example, estradiol, estradiol esters such as estradiol cypionate, ethinyl estradiol, estrone preparations such as conjugated estrogens, and esterified estrogens. Estrogen is available commercially from, for example, Wyeth-Ayerst Laboratories (Philadelphia, PA) and Bristol-Myers Squibb (New York, NY).

## 25 *Pharmaceutical Compositions*

Estrogen and leptin or an analog of leptin can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compounds and compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or

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suspensions in aqueous physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal drops, or aerosols. Compositions for other routes of administration may be prepared as desired using standard methods.

5           Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for  
10 controlling the release of a compound of the invention *in vivo*. Other suitable parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain excipients such as lactose, if desired. Inhalation formulations may be aqueous solutions containing, for example,  
15 polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or they may be oily solutions for administration in the form of nasal drops. If desired, the compounds can be formulated as gels to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration.

          The invention will be further described in the following examples, which  
20 do not limit the scope of the invention described in the claims.

### Examples

**Example 1 - Cell Culture Methods:** Tissue culture media and reagents were either purchased from Sigma Chemical Co. (St. Louis, MO) or GIBCO BRL (Grand Island, NY). Tissue culture plasticware was purchased from Corning  
25 (Corning, NY). Molecular biology reagents and enzymes were purchased from Boehringer Mannheim (Indianapolis, IN).

Conditionally immortalized hMS cell lines were established by transfecting hMS cells with a gene coding for a temperature-sensitive mutant TsA58 of SV40 large T antigen (SV40LTA). Hicok, K. et al., J. Bone Miner.



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Res., 1998, 13:1-13. Six cell lines were characterized and displayed similar phenotypes. The hMS[2-12] cell line was utilized for these studies.

The hMS[2-12] cells were maintained in an humidified atmosphere at 34°C, 5% CO<sub>2</sub>, in standard growth medium including  $\alpha$  minimum essential medium (5  $\alpha$ -MEM) growth medium containing 10% (v/v) heat inactivated fetal bovine serum (HI-FBS), 0.2  $\mu$ g/ml Geneticin (G418), and 1% of stock penicillin (10,000 U/ml)/streptomycin (10,000  $\mu$ g/ml). Medium was changed twice a week. The hMS cell lines were able to concurrently differentiate toward osteoblasts and adipocytes in a standard differentiation medium containing 10% HI-FBS, 10<sup>-8</sup> M dexamethazone, 10<sup>-8</sup> M (Dupont-NEN, Boston, MA), 1,25-dihydroxyvitamin D<sub>3</sub>, 10 mM  $\beta$ -glycerophosphate, and 100  $\mu$ M L-ascorbic acid phosphate (Asc-P) (WAKO Chemicals, USA, Inc., Richmond, VA). All experiments were performed in this differentiation medium in the presence of vehicle 50 mM (disodium phosphate buffer, pH 7.5) or freshly-prepared leptin (Eli-Lilly & Co., Indianapolis, IN), unless otherwise indicated.

All experimental values were expressed as mean  $\pm$  SEM. Two sample Student's t-Test was used to evaluate differences between the stimulated sample and the respective control. Multiple measurement ANOVA was used for dose and time-dependent differences. A P value of 0.05 was considered significant.

20        **Example 2 - Expression of leptin-R:** To evaluate the presence of the leptin-R in the hMS 2-12 cell line, Western blot analysis was performed using a rabbit polyclonal IgG epitope affinity-purified anti leptin-R antibody against the common form of leptin-Rs (ABR, Golden, CO).

Cells were plated at a density of 2x10<sup>4</sup> cells per cm<sup>2</sup> in T75 flasks in growth medium and maintained for 2 days at 34°C. The cells then were washed 25 twice in phosphate buffered saline (PBS) and cultured in differentiation medium at 39.5°C in the absence or in the presence of 0.6  $\mu$ g/ml of rh-lep. After 6 days, the cells were washed twice with PBS, the pellet was suspended in electrophoresis buffer, and electrophoresed through a 7.5% sodium dodecyl sulfate polyacrylamide

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gel (SDS PAGE) under reducing conditions, using a protein mixture (Amersham, Arlington Heights, IL) as standards. The blots were electrotransferred onto a nitrocellulose membrane (Schleicher and Schuell, Reeve, NH). A hematopoietic cell line, K562 was used as a positive control.

- 5           The blots were blocked for 2 h in blocking buffer consisting of PBS (pH 7.4) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA, then probed in blocking buffer with an anti leptin-R antibody (1  $\mu$ g/ml) at 4°C. After 20 h of incubation, the blots were washed twice with PBS containing 0.1% (v/v) Tween 20 and incubated in blocking buffer for 2 h with a peroxidase-conjugated affinity pure  
10 IgG goat anti-rabbit antibody (1:300 final dilution). After three washes in PBS containing 0.1% (v/v) Tween 20, immunoreactive proteins were visualized with an ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL) according to manufacturer's instructions.

- Leptin-R was present in the hms[2-12] cell line when cultured at 34°C  
15 in regular medium. Immunoblotting analysis yielded a 120-kD band in the protein extracts, consistent with the molecular weight of the short form of the leptin-R. No significant difference was observed between 34°C and the control and rh-lep-treated condition at 39.5°C. A 220-kD fragment also was detected in protein extracts of cells at 39.5°C and is consistent with the long form of the receptor.

- 20           Leptin-R gene expression also was detected by reverse-transcriptase PCR (RT-PCR) at both 34°C and 39.5°C. Cells were plated at a density of  $1.8 \times 10^5$  cells per well of 6 well plates in growth medium and maintained 2 to 4 days at 34°C. The cells were washed twice in PBS and cultured for various time intervals in differentiation medium at 39.5°C in the absence or in the presence of 0.15-2.4  
25  $\mu$ g/ml of rh-lep. Total cellular RNA were extracted using the RNA STAT-60™ kit following manufacturer's instructions (TEL-TEST, Inc., Friendwoods, TX). cDNA were synthesized from 2  $\mu$ g total RNA in a 20  $\mu$ l reaction mix containing 1x reverse transcriptase buffer (25 mM Tris HCl, 8 mM MgCl<sub>2</sub>, 30 mM KCl, and 1 mM dithiothreitol (DTT), pH 8.5), 50 pmol of poly-dT, 2 mM each of dATP,

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dCTP, dGTP, and dTTP, 25 U of RNase inhibitor, and 25 U of AMV reverse transcriptase for 1 h at 42°C.

- Amplifications were performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Aliquots of 1  $\mu$ l cDNA were amplified in a 25  $\mu$ l PCR reaction mixture containing 5 pmol of 5' and 3' oligo-primers, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, and 1.5 mM MgCl<sub>2</sub>), 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.25  $\mu$ l of [ $\alpha$ -<sup>32</sup>P] dCTP (10  $\mu$ Ci/ $\mu$ l, Dupont-NEN, Boston, MA), and 0.5 U expanded high fidelity *Taq* DNA polymerase. A 375-bp fragment from a region common to all OB-R variants (sense, 5'-
- 10 TGTTGTGAATGTCTTGTGCC-3', SEQ ID NO:1; antisense, 5'-TACTCCAGTCACTCCAGATTCC-3', SEQ ID NO:2), and a 340-bp fragment specific to the long form variant of the OB-R (sense, 5'-
- ATAGTTCAGTCACCAAGTGC-3', SEQ ID NO:3; antisense, 5'-GTCCTGGAGAACTCTGATGTCC-3', SEQ ID NO:4) were amplified, in
- 15 duplicate, using 30-35 cycles with denaturation at 94°C (30"), annealing at 55°C (30"), and extension at 72°C (30").

- PCR products were analyzed by electrophoresis through a 1.5% (w/v) agarose gel containing 0.01% (w/v) ethidium bromide. Visualized PCR product bands were excised from the gel, and radioactivity within gel slices was quantitated
- 20 using a Beckman LS600 scintillation counter (Beckman Instruments, Fullerton, CA). Quantification of PCR product was normalized to GAPDH PCR products to control for variation in cDNA synthesis efficiency. The cDNA from three separate RNA samples were analyzed for each gene and condition. All gene products were extracted using Wizard® PCR Preps DNA (Promega, Madison, WI). For sequence
- 25 analysis, approximately 150 ng of each purified cDNA fragment was added to 3.2 pmol of either 5' or 3' primer and analyzed in both directions using an automated DNA sequence analyzer.

Using RT-PCR, OB-R gene expression was detected at both 34°C and 39.5°C, with a 2-fold increase observed at the restrictive temperature.

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Administration of leptin did not seem to modulate gene expression or protein synthesis of the leptin-R.

**Example 3 - Effects of Leptin on Cell Proliferation:** Cell proliferation was assessed by [<sup>3</sup>H] thymidine incorporation. Cells were plated at a density of 2x10<sup>4</sup> cells per well of 48 well plates in growth medium. After 48 hours at 34°C, cells were washed twice in PBS, and incubated at 34°C for an additional 24 hours in αMEM + 0.1% (w/v) of BSA, without FBS, in order to synchronize the cell population. Cells then were incubated in differentiation medium in the presence of vehicle or 0.6 μg/ml of leptin for 48 hours at 34°C or 39.5°C. To assess synthesis of DNA, 1 μCi of [<sup>3</sup>H]-thymidine (Dupont-NEN, Boston, MA) was added for the final 24 hours of incubation. After the cells were harvested by trypsinization, thymidine was extracted by trichloroacetic precipitation, and detected by scintillation counting.

As shown in Figure 1, no significant differences in <sup>3</sup>H-thymidine incorporation were observed with leptin administration as compared with vehicle when the cells were cultured at either 34°C or 39.5°C. Results in Figure 1 are representative of three experiments and are expressed as mean ± SEM of quadruplicates. The levels of incorporation confirmed that at 39.5°C, the restrictive temperature for the SV40 large-T antigen, the proliferation rate of the cell line is reduced by 2-fold.

**Example 4 - Effects of Leptin on Gene Expression:** Adipocyte and osteoblast-specific mRNA was quantitated using RT-PCR conditions described in Example 2. Amplifications specific for the following cDNAs were performed: bone/liver/kidney alkaline phosphatase (AP), osteocalcin (OC), type I collagen (Col I); core-binding factor α1 (Osf/Cbfa1), peroxisome proliferator-activated receptor γ<sub>2</sub> (PPARγ<sub>2</sub>), lipoprotein lipase (LPL), adipsin, leptin, and housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) as a control for RNA loading of RT reaction. Primer sequences and amplification profiles used for all of these

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genes were reported previously except for adipsin, leptin, and PPAR $\gamma$ 2. Hicok K. et al., *J. Bone Miner. Res.*, 13:1-13, 1998. Osf/Cbfa1 primers amplified a 267-bp fragment starting at nucleotide 136 of the human cDNA sequence. Komori et al., *Cell*, 89:755-764 (1997). A 251 bp cDNA fragment of adipsin (sense, 5'-

5 GGTCACCCAAGCAACAAAGT-3', SEQ ID NO:5; antisense, 5'-

CCTCCTGCGTTCAAGTCATC-3', SEQ ID NO:6), a 227-bp cDNA fragment of

leptin (sense, 5'-GCTTTGGCCCTATCTTTTCT-3', SEQ ID NO:7; antisense, 5'-

CACGTTTCTGGAAGGCATAC-3', SEQ ID NO:8), and a 390 bp cDNA fragment

of PPAR $\gamma$ 2 (sense, 5'-CAGTGGGGATGTCTCATAA-3', SEQ ID NO:9; antisense,

10 5'-CTTTTGGCATACTCTGTGAT-3', SEQ ID NO:10) were amplified for 30-35

cycles with denaturation at 94°C (30"), annealing at 55°C (30"), and extension at

72°C (30"). Amplification of LPL and Col I were performed for 28 cycles with

denaturation at 94°C (30"), annealing at 55°C (1'), and extension at 72°C (2').

Amplification of AP and OC were performed for 28 cycles with denaturation at

15 94°C (30"), annealing at 55°C (30"), and extension at 72°C (30"). All reactions

ended in a 7 minute incubation at 72°C.

Changes in genotype expression were evaluated in both adipogenic and osteoblastic lineages. As shown in Figures 2A-2C, after 3 days at 39.5°C, rh-lep increased the early osteoblastic differentiation markers AP and type I collagen by

20 66% (p<0.03) and 145% (p<0.001), respectively. The level of OC mRNA, a late

marker, rose last but continued to increase at 6 days, up to 147% (p<0.001). The

osteoblastic marker expression was dose-dependently increased by rh-lep

administration. By contrast, a 40% decrease in adipsin and leptin mRNA

expression was observed at 9 days (p<0.001), compatible with the decrease of late

25 phenotypic differentiation (Figure 3A-3C). Surprising, early markers of adipogenic

differentiation, i.e., PPAR $\gamma$ 2 and LPL, were increased in a dose-dependent manner

by rh-lep administration.

#### **Example 5 - Effects of Leptin on Osteoblastic Phenotypic**

**Differentiation:** AP activity, and OC and Col I secretion were assayed as follows.

- 20 -

Cells were plated at a density of  $2 \times 10^4$  cells per well of 48 well plates in growth medium and allowed to adhere for 48 h at  $34^\circ\text{C}$ . Cells were washed twice in PBS and incubated in differentiation medium at  $39.5^\circ\text{C}$  in the absence or in the presence of 0.15 to  $2.4 \mu\text{g/ml}$  of rh-lep for 3, 6, and 9 days. AP enzyme activity was

5 quantitated in a cell lysate by spectrophotometric measurement of p-nitrophenol release at  $37^\circ\text{C}$  for 1 hour. For other bone-related protein assays, the medium was replaced in all conditions, 24 hours before harvest, with 1 ml of  $\alpha$ -MEM containing 0.1% (w/v) BSA. Conditioned media were collected and measured for OC (Novocalcin, Metra Biosystem, Mountain View, CA) and Col I (Prolagen-C,

10 Metra Biosystem) proteins by ELISA. Levels of leptin protein were measured by RIA. Results were then normalized to total cellular protein values, as measured from cell lysate by Bradford method (Bio-Rad Laboratories, Hercules, CA).

As shown in Figure 4, administration of rh-lep for 3 days at  $39^\circ\text{C}$  increased AP activity of the cells in a time ( $p < 0.02$ ) and dose-dependent ( $p = 0.002$ )

15 manner as compared with standard growth medium. A 24% increase in AP activity was observed after 3 days in culture and a 42% increase after 9 days in culture as compared to control. As indicated in Figures 5A-5B, levels of type I procollagen and osteocalcin proteins released in the medium also were significantly increased with time (72% ( $p < 0.01$ ) and 37% ( $p < 0.01$ ), respectively).

20 Mineralization is the final step of osteoblastic differentiation and was assessed by Alizarin Red S staining. Vacca, L.L., Calcification Laboratory Manual of Histochemistry, Raven Press, New York, NY, pp. 334-336. Cells were plated at a density of  $8 \times 10^4$  cells per well of 12 well plates in growth medium and allowed to proliferate for 4 days at  $34^\circ\text{C}$ . The cells then were incubated at  $39.5^\circ\text{C}$  in

25 differentiation medium in the absence or in the presence of  $0.6 \mu\text{g/ml}$  of rh-lep for 22 days. Medium containing freshly prepared rh-lep was renewed every three days. Cells were fixed in 70% ethanol for 1 hour at room temperature, then washed with PBS and stained with 40 mM Alizarin Red S, pH 4.2 for 10 minutes at room temperature. Cell preparations then were washed 5 times with deionized water and

30 incubated in PBS for 15 minutes to eliminate non-specific staining. The stained

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matrix was assessed using a Nikon Diaphot inverted microscope and photographed by a Nikon 35 mm camera (Nikon Corp, Tokyo, Japan). Alizarin Red S staining was released from the cell matrix by incubation in cetyl-pyridinium chloride for 15 minutes. The amount of released dye was quantified by spectrophotometry at 540 nm. Bodine, P.V.N., et al., J. Bone Miner. Res., 1996, 11:806-819.

As shown in Figure 6, leptin induced a significant 59% increase ( $p < 0.001$ ) in mineralization of the matrix in long-term culture.

**Example 6 - Effects of Leptin on Lipid Accumulation:** Cytoplasmic inclusions of neutral lipids were assessed by Oil Red O staining. Cells were plated at a density of  $5 \times 10^4$  cells per well of 12 well plates in growth medium and allowed to proliferate for 4 days at  $34^\circ\text{C}$ . Cells then were incubated in the differentiation medium at  $39.5^\circ\text{C}$  in the absence or in the presence of  $0.6 \mu\text{g/ml}$  of rh-lep for 6, 9, 12, and 15 days. Cells were observed using a Nikon Diaphot inverted microscope and a Nikon 35 mm camera. The percentage of Oil Red O positive cells was determined blindly by counting cells in 30 contiguous fields per well with a random start.

As shown in Figure 7, administration of rh-lep decreased triglyceride accumulation in hMS[2-12] cells. While stained lipid droplets consistently appeared after 6 days at  $39.5^\circ\text{C}$  in both groups, leptin treatment progressively prevented lipid accumulation. The difference in lipid accumulation between conditions increased with time, from 9-15 days of leptin treatment ( $p < 0.02$ ).

**Example 7 - Effects of Leptin on PPAR $\gamma$ 2 Expression:** Because of the changes of PPAR $\gamma$ 2 mRNA expression, PPAR $\gamma$ 2 protein synthesis induced by rh-lep administration was evaluated by western-blot analysis. Immunoblotting showed a 58 kD band consistent with the PPAR $\gamma$ 2 molecular weight. Treatment by leptin induced a 2-fold increase in PPAR $\gamma$ 2 protein level.

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**Example 8 - Osteogenic Activity of Leptin in Rats:** The effect of long-term leptin therapy on bone loss prevention can be assessed by administering leptin to lean, ovariectomized (OVX) rats. Estrogen-deficiency is induced by removing the ovaries.

- 5           Sixty-two Sprague-Dawley rats were obtained from Charles River laboratories (Portage, MI) at 6 months of age and weighing  $283 \pm 5.1$  g (mean  $\pm$  SEM). Bilateral ovariectomy (n=56, OVX) or sham surgery (n=8, intact) was performed at 5 months and 3 weeks of age. At four days postsurgery, OVX rats were stratified by weight and randomized to a baseline (n=8) and one of five
- 10 groups (8 rats per group): 1) leptin, 2) 17-alpha-ethinyl estradiol, 3) leptin plus 17-alpha-ethinyl estradiol, 4) vehicle, and 5) pair fed to leptin treated group. Treatments were not started until one week post-OVX to allow estrogen levels to become depleted. Subcutaneous administration of leptin was performed through an Alzet osmotic pump. A 0.35 mg/kg/day dosage of leptin was used in these
- 15 experiments, with a daily delivery rate of 0.12 mg/rat/day. This dosage was chosen based on the physiological effect of modulating food intake in rats. In order to maintain a continuous leptin infusion rate, new osmotic pumps were implanted after two weeks of therapy. The dosage of 17-alpha-ethinyl estradiol was 200  $\mu$ g/kg/day.
- 20           Fluorochromes to label mineralization bone matrix were administered by juxta tail vein injection 1 day before starting treatment (calcein-tetracycline-HCL, 20 mg/kg of body weight, Sigma), 10 days before sacrifice (tetracycline, Sigma; 20 mg/kg of body weight), and 3 days before sacrifice (calcein-tetracycline, as described for day 1). The two fluorochrome labels were readily differentiated
- 25 under UV illumination, as tetracycline fluoresces pale yellow, while calcein fluoresces bright green.

Histomorphometric measurements were performed with an osteomeasure semiautomatic analysis system (Osteomeasure Instruments, Inc. Atlanta GA) consisting of a computer coupled to a photomicroscope and image analysis system.

30 In this system, a high-resolution color video camera records the image of the



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specimen through the microscope (Olympus BH-2, New Hyde Park, NY) and displays the image on a video monitor that registers the movement of a digitizing pen on a graphics tablet. As the pen is moved along the graphics tablet, a tracing appears superimposed on the image of the specimen displayed on the video screen.

- 5 The region of interest was traced. The line lengths and area bounded by the lines then are calculated by the computer.

Ground transverse sections were used for histomorphometric analysis of cortical bone. Cross sections 150  $\mu\text{m}$  thick were cut at a site just proximal to the tibia-fibula synostosis with a low-speed saw (Isomet, Buehler, Lake Bluff, IL) equipped with a diamone wafer blade. The sections were ground to a thickness of 15-20  $\mu\text{m}$  on a roughened glass plate and mounted in glycerin before microscopic examination under UV illumination to visualize fluorochrome labeling. The following were performed: cross-sectional area, defined as the area of bone and marrow cavity bounded by the periosteal surface of the specimen; medullary area, defined as the area delineated by the endocortical surface of the specimen; cortical bone area calculated as the difference between the cross-sectional and medullary area; periosteal perimeter, defined as the total perimeter enclosing the cross-section (periosteal perimeter includes fluorochrome-labeled and non-labeled perimeters); periosteal bone formation rate, calculated as the area bounded by tetracycline labels divided by the labeling period of 21 days; and periosteal mineral apposition rate (MAR), defined as the periosteal bone formation rate divided by the labeled perimeter.

After fixation for a minimum of two days in 70% ethanol, the proximal tibial metaphysis was dehydrated in a series of increasing concentrations of ethanol and embedded without demineralization in a mixture of methylmethacrylate-2-hydroxyethyl-methacrylate. The embedded tibias were sectioned at an indicated thickness of 5 microns on the Reichert-Jung Supercut 2050 microtome to obtain midcoronal sections. The sampling site used was 1 mm distal to the growth plate in the secondary spongiosa and extended bilaterally in each section, excluding the endocortical surfaces.

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Bone formation rate was calculated as the product of the double label surface and mineral apposition rate. The mineral apposition rate, expressed as microns per day was the mean distance between the tetracycline and calcein label, divided by the labeling interval of 10 days. Double labeling surface was  
5 determined as the bone surface with tetracycline and calcein label. Dynamic measurement related to bone resorption. Calcein label perimeter was measured in a growth-adjusted metaphyseal sampling site, and subsequent resorption was calculated as described by Westerlind, K.C. et al., J. Bone Miner. Res., 1998, 13:1023-1031.

10 Analysis of variance was performed with post hoc Newman-Keuls multiple comparison testing.  $P < 0.05$  was considered significant.

As indicated in Figure 8, OVX animals gained weight when compared with the sham operated group. Figure 9 provides the average daily food intake of OVX animals. OVX-induced weight gain was prevented by the administration of  
15 estrogen. The leptin, vehicle treated, and pair fed groups gained a statistically significant amount of body weight compared to the sham operated, estrogen, or estrogen + leptin treated groups. The leptin treated group had a significantly lower body weight at the end of the study, compared with the vehicle treated group; but was not significantly different from the pair fed group. Thus, the dose and route of  
20 administration used for leptin treatment were effective in modulating appetite and slightly impaired weight gain. The leptin treated group body weight, however, was intermediate between the vehicle and estrogen treated groups, avoiding any bias due to starvation on further analysis on bone metabolism. As expected, both the leptin and leptin + estrogen treated group had significantly higher levels of plasma leptin  
25 levels as compared with control. Leptin CSF levels also were measured, with no statistically significant differences observed among the different groups (Table 1).

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**TABLE 1**  
**Leptin Plasma and CSF Level (ng/ml)**

Day	Plasma			CSF	
	0	15	30	0	30
Control	1.51 ± 0.23	--	--	0.19 ± 0.02	--
Vehicle		1.31 ± 0.17	1.14 ± 0.10		0.20 ± 0.00
Leptin		2.23 ± 0.23	2.77 ± 0.39		0.22 ± 0.05
Estrogen		1.52 ± 0.14	1.52 ± 0.20		0.23 ± 0.03
Leptin + Estrogen		1.91 ± 0.25	2.75 ± 0.66		0.19 ± 0.02
Pair Fed		1.50 ± 0.21	1.74 ± 0.21		0.20 ± 0.03
Sham		1.42 ± 0.09	1.19 ± 0.14		0.18 ± 0.05

As indicated in Figure 10, cancellous bone volume (bone volume/total volume, BV/TV) was significantly decreased 1 month after OVX. BV/TV was expressed as % and represents the % of the assessed volume that is occupied by bone, with the cortical shell being excluded from measurement. The difference is the bone marrow space. This decrease in BV/TV was prevented by estrogen treatment, as measured by histomorphometry analysis. Leptin treatment also significantly reduced the BV/TV, although it was not as effective as estrogen treatment. The combination of leptin and estrogen did not further increase BV/TV, but was more effective than leptin alone. Leptin treatment preserved trabecular bone architecture by maintaining trabecular number (Tb.N.). Trabecular bone volume of treated animals is shown in Figure 11. Trabeculae thickness (Tb.Th) was not significantly different when compared with vehicle treated group.

The number of osteoclasts (Oc.S/BS, wherein "BS" is bone surface) and osteoblasts (Ob.S/BS, MS/BS, wherein "MS" is mineralization surface), and the activity of osteoblasts (MAR, BFR/BS) were not different between leptin and vehicle treated groups (Table 2). The combination of leptin and estrogen, however, further decreased the number of osteoblasts (Ob.S/BS, MS/BS), when compared to both the estrogen treated group and sham operated group. At the cortical level, leptin therapy prevented the OVX-induced increase in bone formation rate, as did

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estrogen therapy (Figure 12). The combination of estrogen and leptin further decreased the bone formation rate at the cortical level compared with estrogen treated OVX rats and shams. This effect was secondary to a decrease in MAR of the sham operated group, whereas other groups had a 2-fold increase in MAR.

5

TABLE 2

	Oc.s/BS %	Ob.s/BS %	LS/BS %	MAR $\mu\text{m}/\text{day}$	BFR/BS $\mu\text{m}^3/\mu\text{m}^2/\text{d}$
Baseline	$8.9 \pm 1.2$	$3.5 \pm 1.7$	--	--	--
Vehicle	$9.8 \pm 1.1$	$10.5 \pm 1.6$	$14.8 \pm 2.0$	$0.90 \pm 0.05$	$52.2 \pm 9.4$
Sham	$7.1 \pm 1.3$	$6.2 \pm 1.9$	$4.2 \pm 1.0$	$0.88 \pm 0.03$	$13.6 \pm 3.3$
Estrogen	$6.5 \pm 1.1$	$1.5 \pm 0.7$	$4.2 \pm 1.2$	$0.83 \pm 0.06$	$13.8 \pm 4.8$
10 Estrogen + Leptin	$5.8 \pm 1.1$	$0.4 \pm 0.3$	$1.9 \pm 0.6$	$0.93 \pm 0.15$	$5.8 \pm 1.6$
Leptin	$10.4 \pm 1.0$	$11.6 \pm 3.1$	$13.8 \pm 2.2$	$0.99 \pm 0.07$	$51.3 \pm 9.7$

The high levels of circulating leptin may be sensed by an early bone marrow precursor as a signal that extra fat is being accumulated, and bone formation is needed to sustain this extra fat. It seems reasonable to believe that, in addition to these effects, leptin may play a role informing and preparing the bone for the mechanical stress that puberty and obesity will impose. It could be postulated from these data that leptin interferes with the fatty acid effect on adipocyte differentiation; an autocrine leptin action on early progenitor cells, could be an useful mechanism to inform and prepare the bone of the extra-weight and stimulate bone differentiation.

#### Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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**Claims**

What is claimed is:

1. A method of inducing differentiation of a bone marrow stromal progenitor cell to an osteoblastic lineage comprising contacting said bone marrow  
5 stromal progenitor cell with an amount of leptin or an analog thereof effective to induce said differentiation.
2. The method of claim 1, further comprising monitoring markers of osteoblast differentiation.
3. A method of inducing bone formation in a mammal comprising  
10 administering an amount of leptin or an analog thereof effective to induce said bone formation.
4. The method of claim 3, further comprising monitoring said mammal for markers of osteoblast differentiation.
5. The method of claim 3, further comprising monitoring said  
15 mammal for beneficial changes in bone mineral density or bone structure.
6. The method of claim 3, wherein estrogen is administered with leptin or an analog thereof.
7. An article of manufacture comprising packaging material and a pharmaceutical agent within said packaging material, wherein said pharmaceutical  
20 agent is therapeutically effective for treating osteoporosis in a patient, wherein said packaging material comprises a label that indicates said pharmaceutical agent is useful for treating osteoporosis in said patient, and wherein said pharmaceutical agent comprises leptin or an analog thereof.

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8. The article of manufacture of claim 7, wherein said pharmaceutical agent further comprises estrogen.

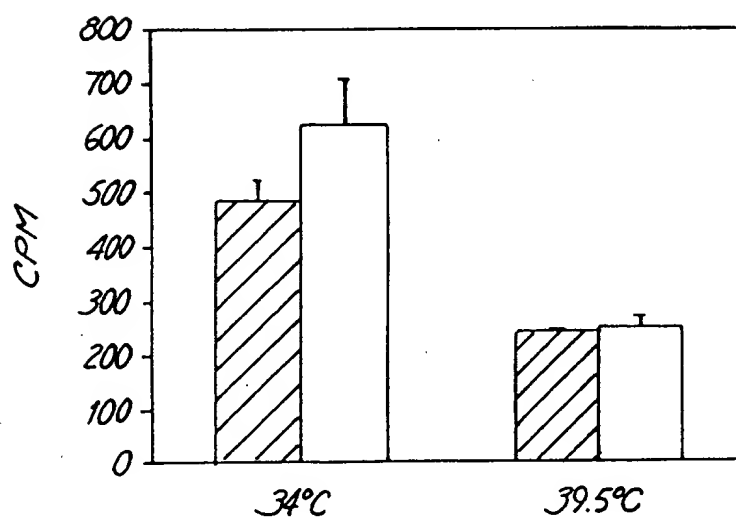
9. A pharmaceutical composition, wherein said composition comprises estrogen, leptin or an analog thereof, and a pharmaceutically acceptable  
5 carrier.

10. Use of leptin or an analog thereof in the manufacture of a medicament for the treatment of osteoporosis.

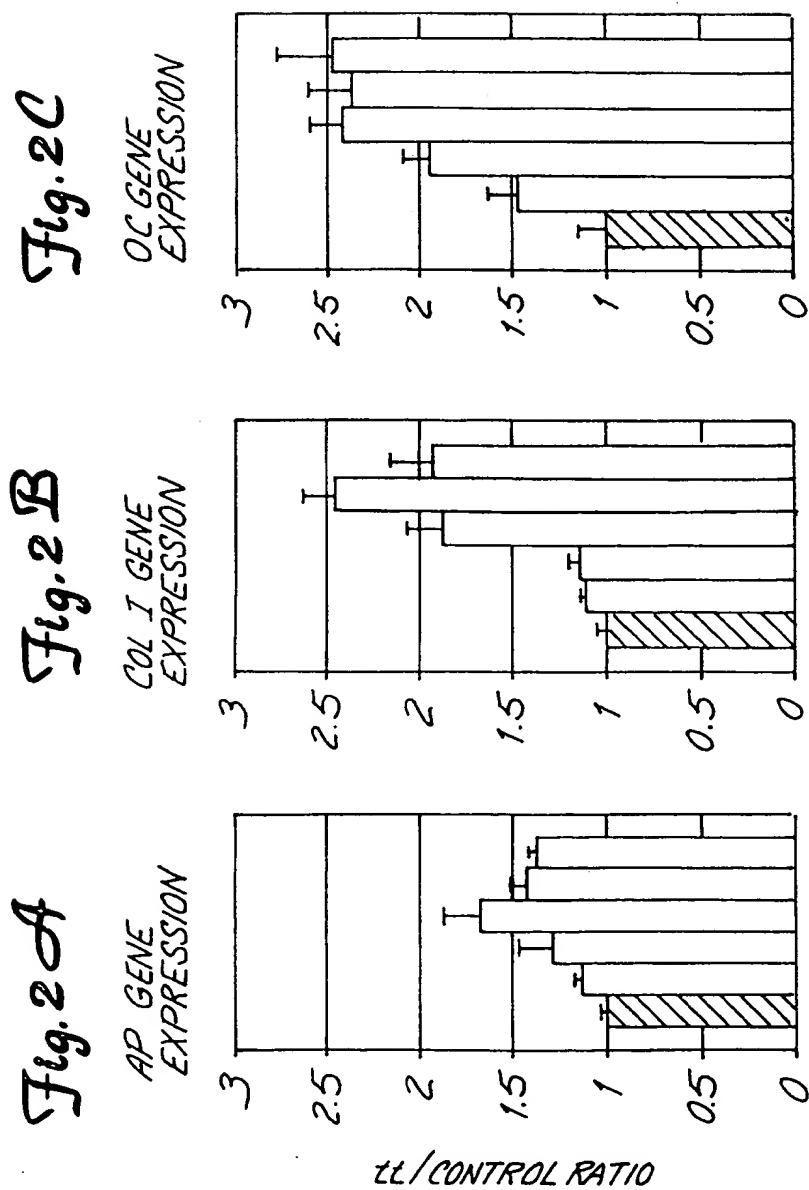
1/12

*Fig. 1*

*EFFECT OF LEPTIN ON hMS[2-12] PROLIFERATION AT 34°C  
AND 39.5°C ASSESSED BY 3H-THYMIDINE INCORPORATION*

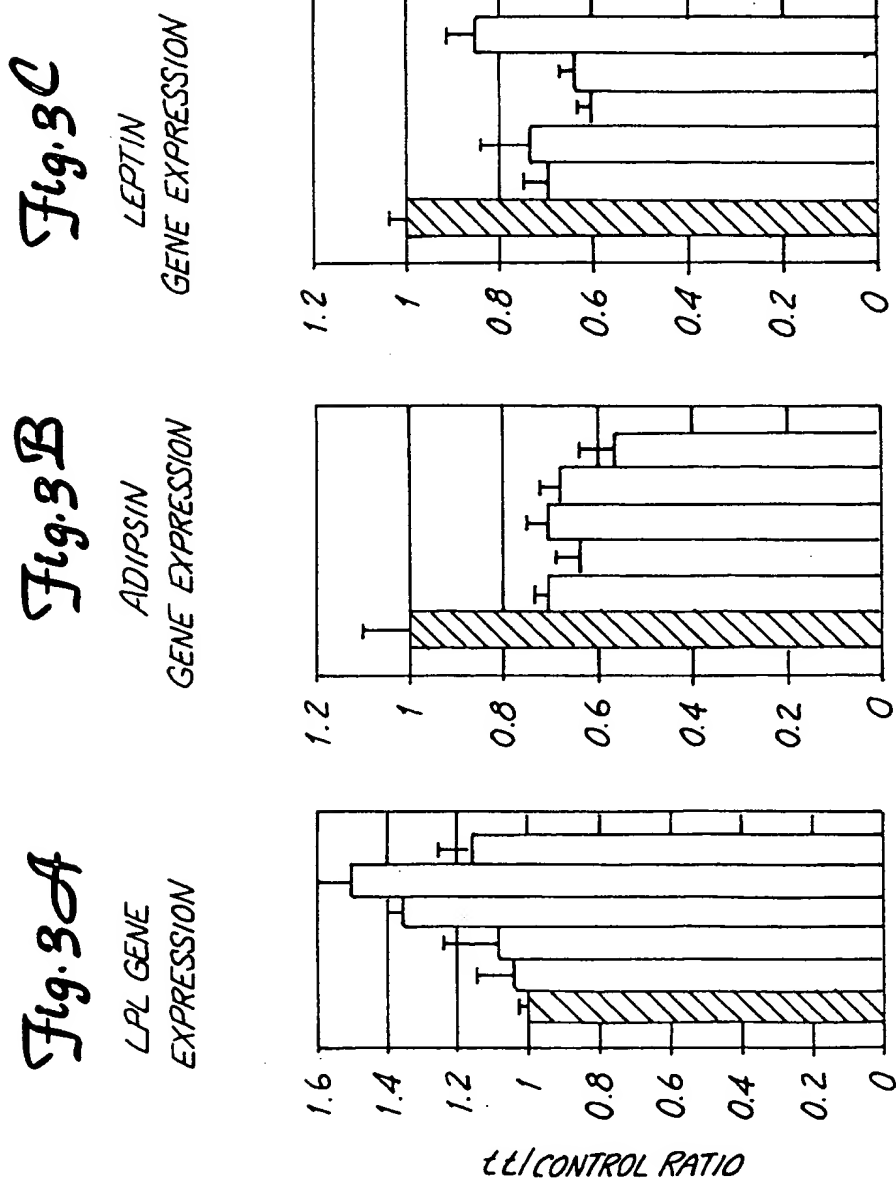


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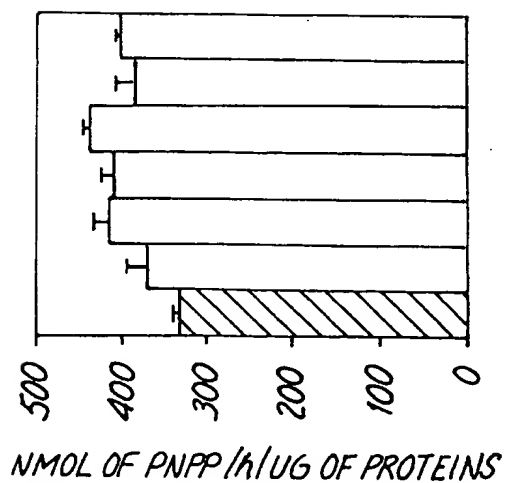
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4/12

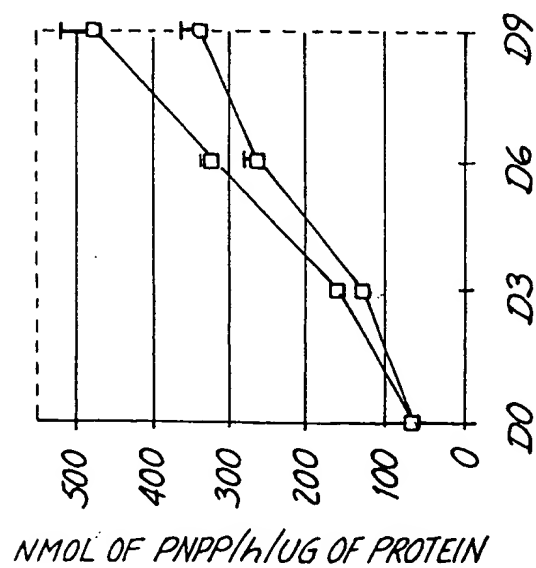
**Fig. 4B**

EFFECT OF LEPTIN ON AP  
ACTIVITY: DOSE-RESPONSE AT D-3



**Fig. 4A**

EFFECT OF LEPTIN ON AP  
ACTIVITY: TIME COURSE



5/12

Fig. 5B

OC EXPRESSION

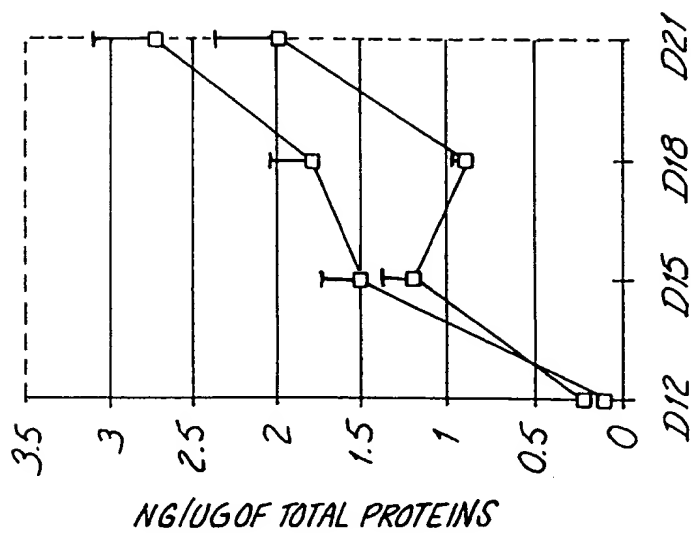
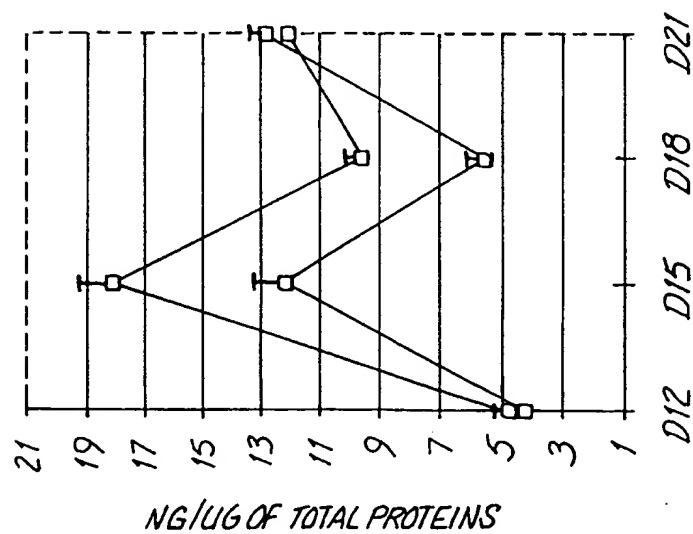
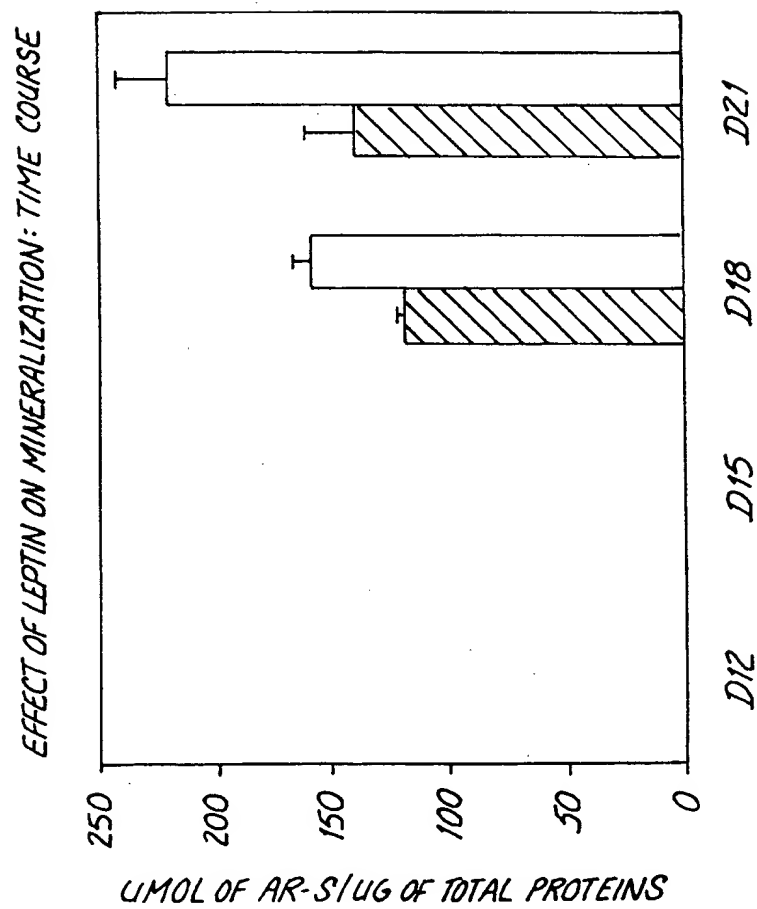


Fig. 5A

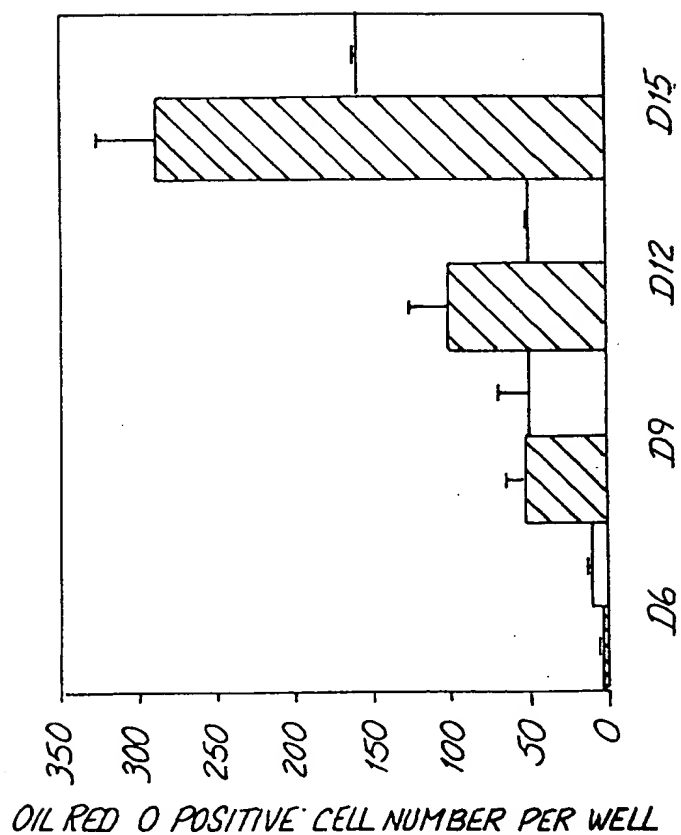
COL I EXPRESSION



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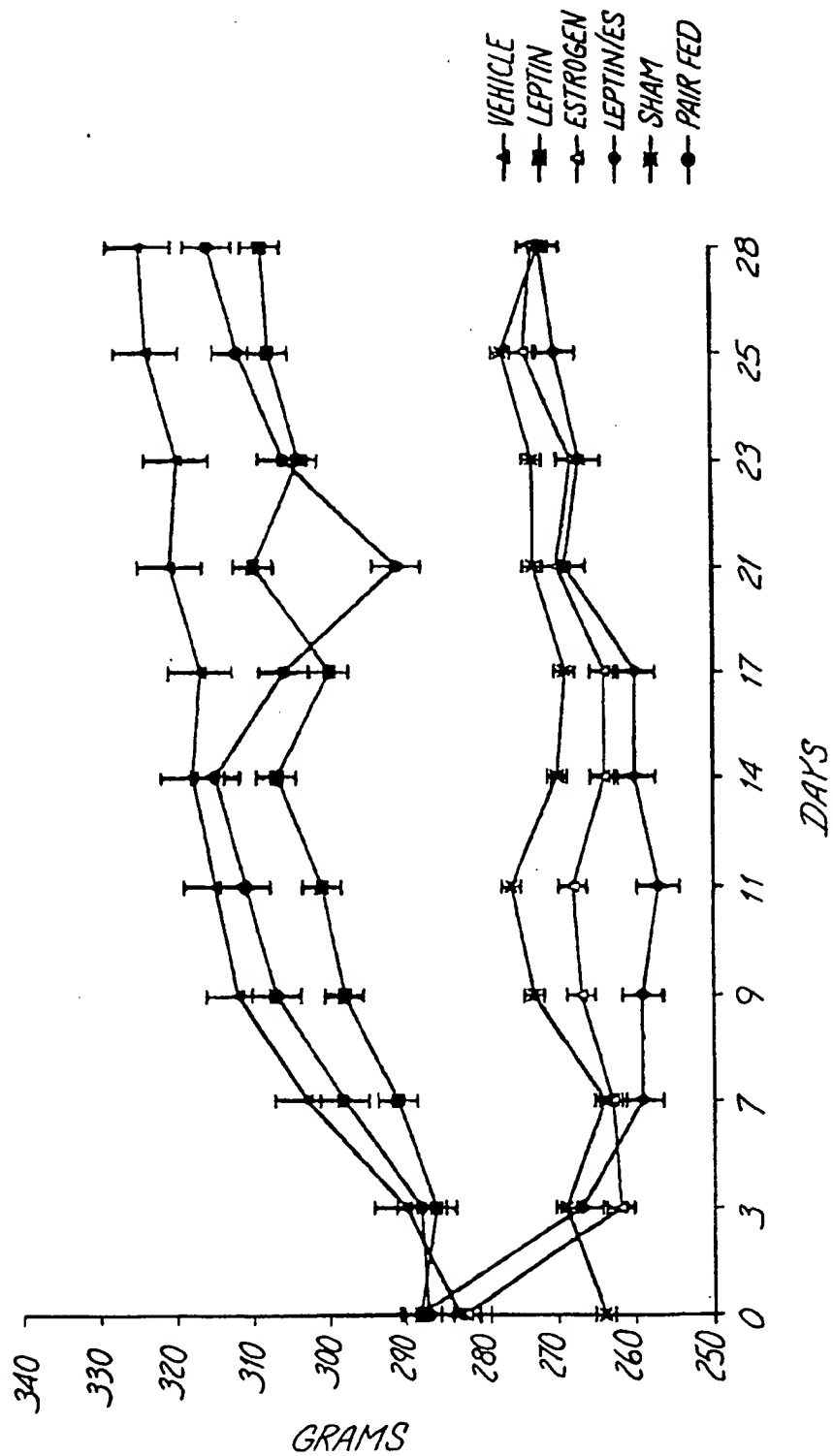
**Fig. 6**

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**Fig. 7***EFFECT OF LEPTIN ON LIPID DROPLET ACCUMULATION: TIME COURSE*

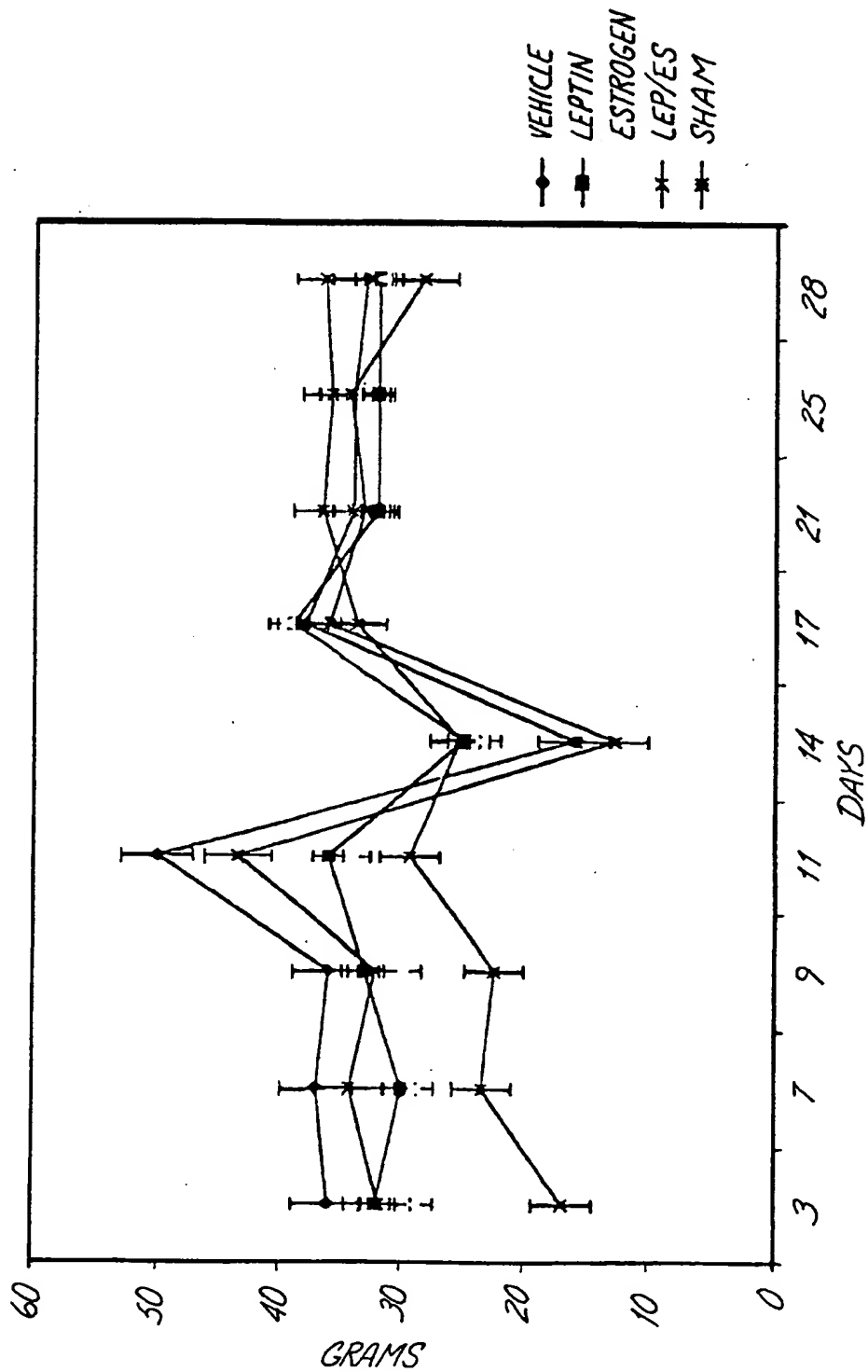
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Fig. 8



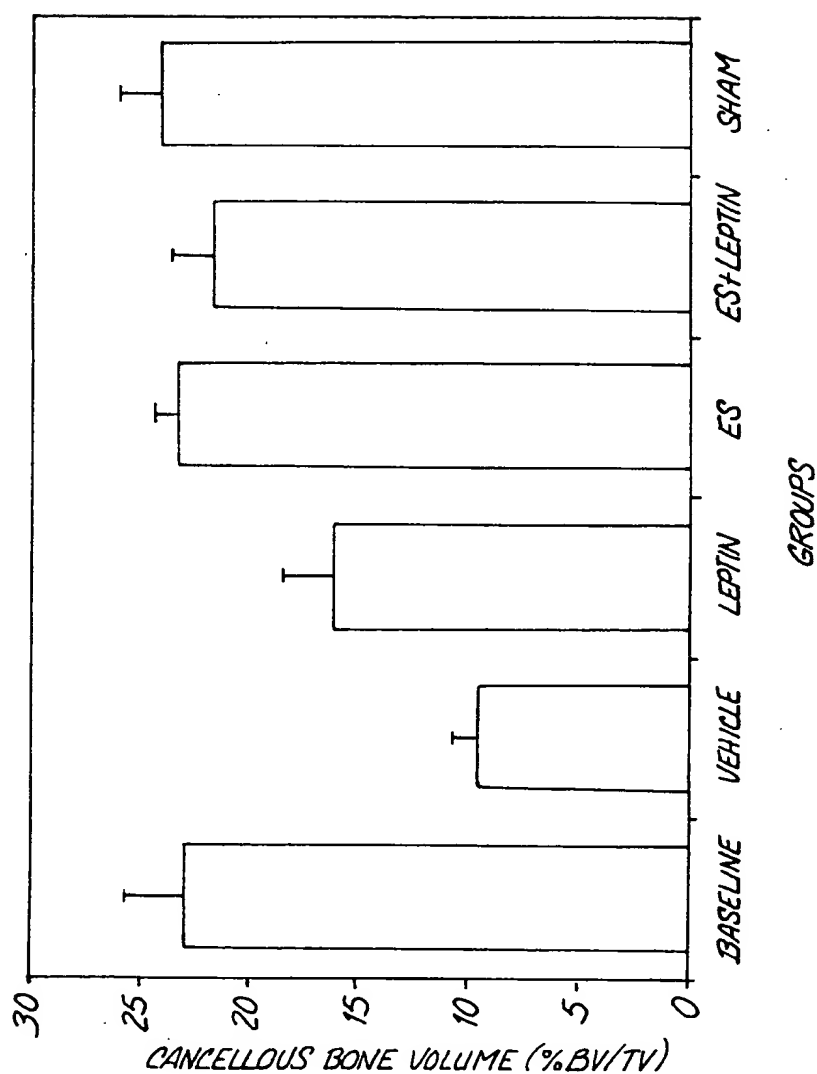
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Fig. 9



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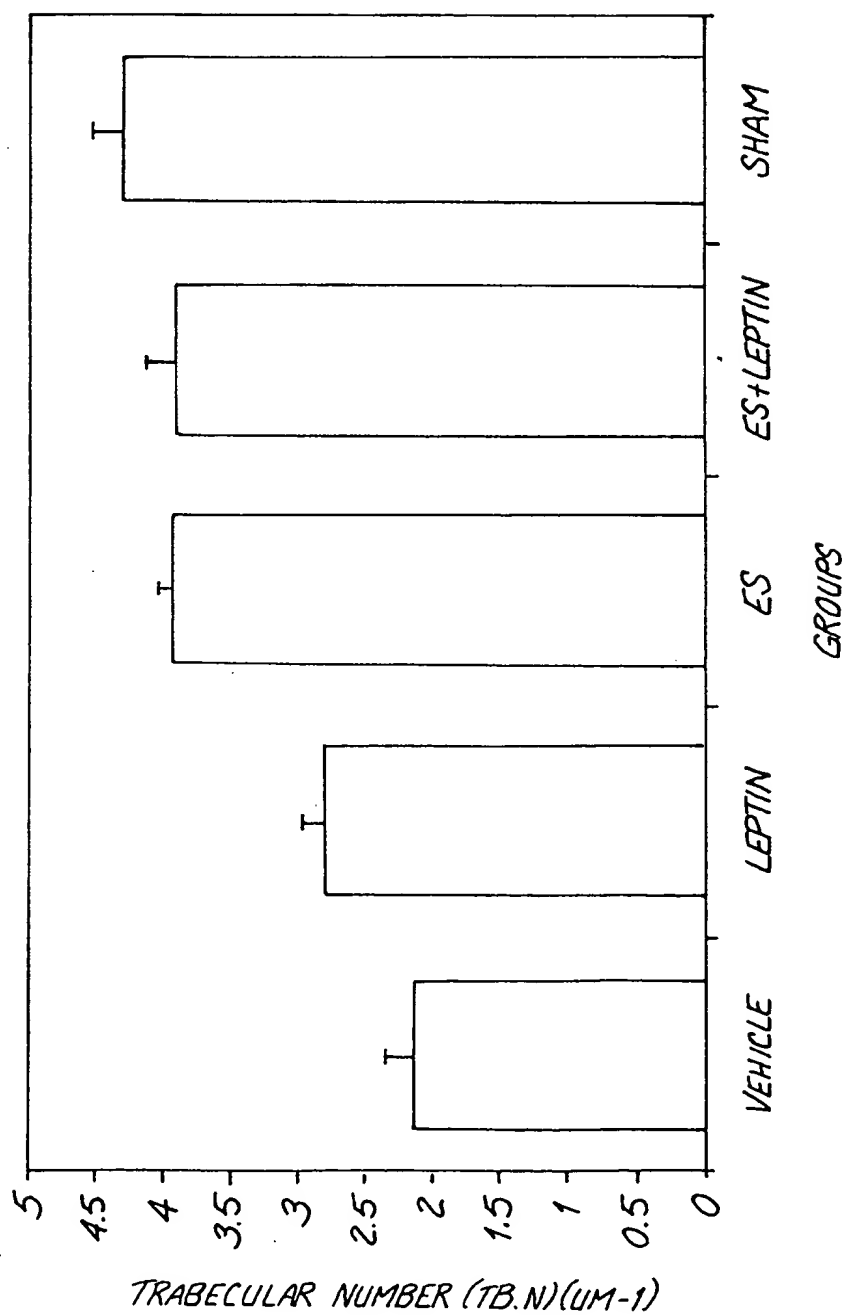
Fig. 10





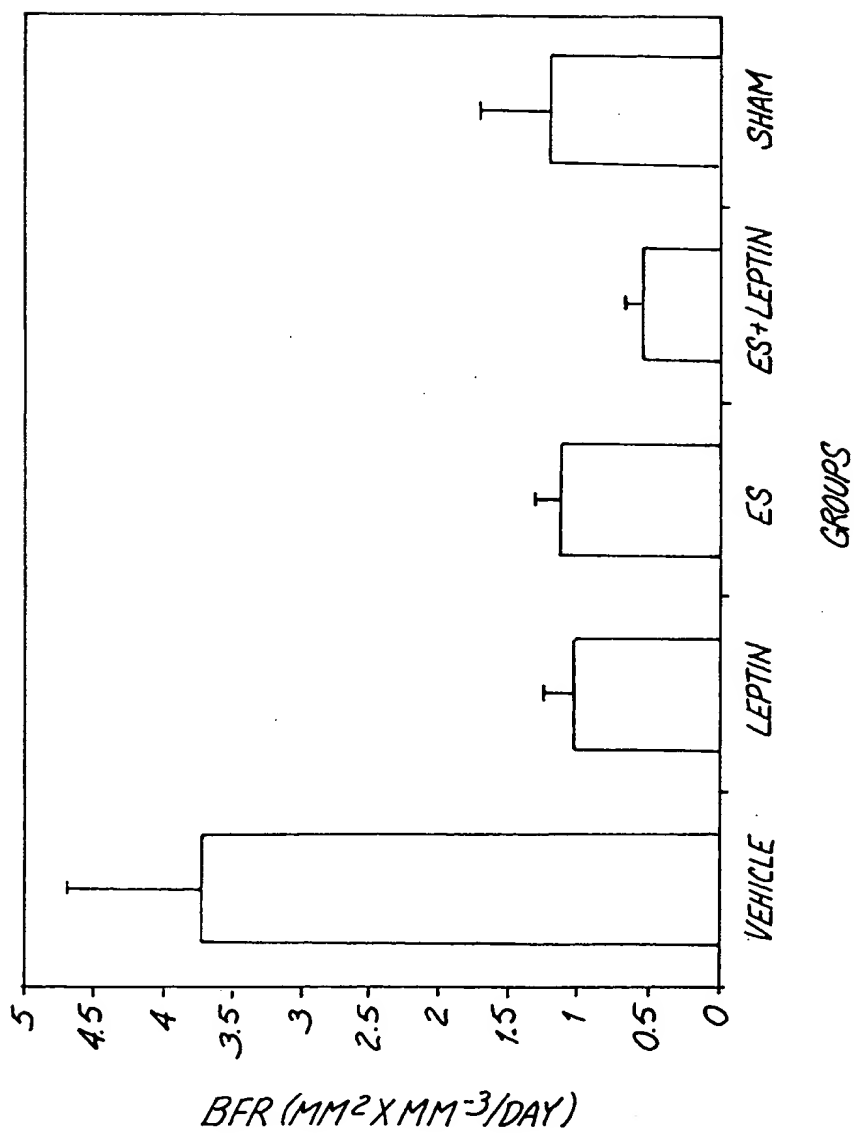
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Fig. 11



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Fig. 12



## SEQUENCE LISTING

<110> Burguera, Bartolome  
Riggs, B. Lawrence  
Thomas, Thierry

<120> TREATMENT OF OSTEOPOROSIS WITH LEPTIN

<130> 07039/150WO1

<150> 60/082,320

<151> 1998-04-20

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/08604

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/00, 38/18, 38/19

US CL : 514/2, 8, 12, 21, 908; 424/85.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8, 12, 21, 908; 424/85.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Database search for leptin/Ob and treat/induce bone marrow stromal progenitor cells, or osteolast

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---,E Y	THOMAS et al. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. Endocrinology, April 1999, Vol. 140, pages 1630-1638, see all.	1-10
X --- Y	WO 97/39767 A1 (ZYMOGENETICS, INC.) 30 October 1997 (30-10-97), see the claims.	1-7,10 ----- 1-10
Y	GAINSFORD et al. Leptin can induce proliferation, differentiation and functional activation of hemopoietic cells. Proc. Natl. Acad. Sci. December 1996, Vol. 93, pages 14564-14568, see all.	1-10
Y	WO 97/32022 A2 (AMGEN INC.) 04 September 1997 (04-09-97), see all.	1-10

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 28 JULY 1999	Date of mailing of the international search report 17 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GARNETTE D. DRAPER Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/08604

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97/18833 A1 (AMGEN INC.) 29 May 1997 (29-05-97), see all.	1-10
A, P	WO 98/18486 A1 (ICOGEN CORP.) 07 May 1998 (07-05-98), see the abstract and claims.	1-10
A	MIKHAIL et al. Leptin stimulates fetal and adult erythroid and myeloid development. Blood, 01 March 1997, Vol. 89, No. 5, pages 1507-1512, see all.	1-10